1.0

1.1

1.25

2.8
3.2
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MICROCOPY RESOLUTION TEST CHART
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CONTROLLED RELEASE OF ANTIBIOTICS FROM BIODEGRADABLE
MICROCAPSULES FOR WOUND INFECTION CONTROL

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INTRODUCTION

Improved methods to prevent and treat infection in contaminated
wounds following traumatic injury are of military significance. Combat
wounds are characterized by a high incidence of infection due to the
inevitable presence of devitalized tissue and foreign body contaminants.
Infection in these wounds is most effectively suppressed the first four
hours after injury when an increased blood supply transports phagocytic
cells and bactericidal factors to the injured area.(1) After four hours,
vascular permeability decreases sharply and a fibrous coagulum forms at
the base of the granulating wound which blocks antibiotics in the serum
from penetrating the area.(2) To control infection, systemic antibiotics
must be administered early when circulation is optimal. If treatment is
delayed, a milieu for bacterial growth is established and complications
associated with established infections occur.(3) Once infections are
established, it becomes difficult to administer systemic antibiotics for an
extended time, at levels that are safe, yet effective at the wound site.
An ideal mode of antibiotic delivery to a contaminated wound would have the
following characteristics: (1) local application in a single dose would
be possible; (2) it would provide an initial burst of antibiotic for
immediate tissue perfusion; and (3) it would provide a sustained and
effective tissue level at the wound site. An antibiotic delivery system
that fulfills these criteria is currently being developed and evaluated.
Unless topically active, drugs are distributed through the body in
plasma, and the amount of drug that hits its target, is only a small part
of the total drug in the body. This ineffective use of the drug is com-
pounded in the trauma patient by hypovolemic shock, which produces a low
perfusion rate of blood to tissues.(4) The ability to administer con-
trolled quantities of a drug to a local area for a sustained time, via a
biocompatible, biodegradable vehicle, provides an opportunity to evaluate
a potentially advantageous method of antibiotic delivery to contaminated

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or infected tissue. Historically, the first success with topical anti-
biotics occurred at Pearl Harbor in 1941 when promptly debrided wounds,
which were sprinkled with sulfanilamide, yielded low infection rates.
Following inappropriate use, however, concretions of sulfa formed in the
tissue causing infection rates to rise.(5) Such failures in the use of
topical antibiotics are now believed to have occurred because improper
drugs were used, or the method of administration failed to release drugs
in an effective concentration. In recent years, topical antibiotics have
been shown to be lifesaving in the prophylaxis and treatment of burn
infections because the antibiotic can be applied repeatedly to the infected
area, assuring a constant source of drug. The role of topical antibiotics
in deep tissue wounds, however, has had less dramatic success. In con-
trast to burn therapy, an infected, deep tissue wound cannot be repeatedly
treated with topical antibiotics unless the infected area is surgically
re-exposed. The effect of single doses of antibiotics, in the form of
powders, sprays, or lavages, are short-term because they are rapidly
removed from the site and excreted. Nevertheless, studies have shown
repeatedly that when risk of infection is high, topical antibiotics
applied in this way do have an effect in reducing infection rates.(6-9)
Until the recent development of sustained release systems, the opportunity
to locally release drugs in tissues for sustained periods was not possible.
Consequently, the theoretical advantages of such a system could not be
evaluated.

The ability to control the release of an antibiotic at the wound site
assures direct contact between effective antibiotic levels and the infect-
ing microorganisms for a sustained period of time. Many drugs have a
therapeutic range, above which they are toxic and below which they are
ineffective. Oscillating drug levels that are commonly observed following
systemic administration may cause alternating periods of ineffectiveness
and toxicity. A sustained-release preparation can maintain the drug in
the desired therapeutic range by means of a single dose. The presence of
nonviable tissue, decreased blood flow subsequent to shock, and nonspecific
drug binding to body proteins while en route to targeted sites, all
decrease the amount of a drug that will reach a wound when the antibiotic
is administered systemically. The local delivery of sustained, therapeutic
amounts of antibiotic theoretically minimizes these problems. Furthermore,
it would be unnecessary to administer multiple doses of potentially toxic
antibiotics. Drugs with short half-lives might be used more efficiently
and undesirable side effects would be minimized.

It is the goal of this study to develop microcapsules that slowly
release effective therapeutic doses of antibiotics in a wound over a
14-day period, by which time the microcapsules will have been biodegraded.
It is the purpose of this paper to report the *in vivo* results obtained
using recently formulated prototype microcapsules.

All initial efforts in the development of the controlled antibiotic
release system have used ampicillin with lactide/glycolide copolymer as

the drug vehicle. This copolymer is ideally suited for *in vivo* drug release since it elicits a minimal inflammatory response, is biologically compatible, and degrades under physiologic conditions.(10) The degradation products are nontoxic and readily metabolized. All microcapsules currently formulated exist as free-flowing microspheres (<250 microns in diameter) consisting of ampicillin anhydrate coated with a poly (DL-lactide-co-glycolide) excipient having a lactide:glycolide ratio of 68:32. Microcapsules of this size can be administered directly to a wound by a shaker-type dispenser or aerosol spray. The rate of biodegradation is controllable because it is related to the molar ratios of the constituent polymers and to the surface area of the microcapsules.

MATERIALS AND METHODS

Microencapsulation Process

Both solvent evaporation and phase separation methods were used in formulating the microcapsules.(11) The microencapsulation process will be described in detail in a subsequent publication, therefore, only a brief description of the process follows. In the solvent evaporation process, ampicillin anhydrate (Bristol Laboratories) was suspended in a polymer solution prepared by dissolving poly (DL-lactide-co-glycolide) in an acetone and methylene chloride solvent. This mixture was then added to a stirred aqueous solution of poly (vinyl alcohol) to form a stable oil-in-water emulsion. The oil microdroplets which formed contain drug, polymer, and polymer solvent. Removal of the polymer solvent by evaporation resulted in solid microcapsules. The poly (DL-lactide-co-glycolide) was synthesized from DL-lactide and glycolic acid. In the phase separation method the antibiotic was suspended in a dilute polymer solution. A non-solvent for both polymer and drug was added to precipitate the polymer onto suspended drug particles in order to produce the microcapsules.

Analytical Procedures Used to Characterize the Microcapsules

Before *in vivo* testing of the microcapsules, the antibiotic content (core load) and *in vitro* release kinetics were evaluated. The core loads were determined by dissolving milligram quantities of microcapsules in methylene chloride and extracting the antibiotic with four volumes of water. The drug dissolved in the water was assayed by direct spectrophotometry, ninhydrin-based colorimetry, or microbiologic techniques. The study of the *in vitro* release rate of the antibiotic was performed by placing known amounts of microcapsules in flasks containing deionized water and agitating at 37°C. Aliquots periodically removed from the receiving fluid were assayed for drug content. A reagent prepared as a ninhydrin-hydrinatin solution was used in a colorimetric assay to evaluate the antibiotic content of the receiving fluid. Reactions of this reagent

with antibiotic solutions of various concentrations developed a color proportional in intensity to the antibiotic content.

Using both microencapsulation processes, ^{14}C-labeled ampicillin anhydrate microcapsules were synthesized.(12) These radiolabeled microcapsules provided an accurate method for determining ampicillin core loadings and *in vitro* release profiles.

In Vivo Evaluation of Microcapsules

Ampicillin microcapsules formulated by both the solvent evaporation and phase separation processes were evaluated *in vivo* to determine the effect of the locally released drug on artificially induced wound infections. Experiments were performed on male Walter Reed strain albino rats, weighing 250-300 grams, that were anesthetized with sodium pentobarbital. The right hind leg was razor-shaved, scrubbed with Betadine, and swabbed with 70% isopropyl alcohol. A wound 2.5 to 3.0 cm in length and 5.0 mm deep was made in the thigh muscle, after which, 0.2 g of sterile dirt was added. The muscles were traumatized by pinching uniformly with tissue forceps, and inoculated with known quantities of *Staphylococcus aureus* ATCC 6538P and *Streptococcus pyogenes* ATCC 19615. The artificially contaminated wounds were treated by layering sterile, preweighed amounts of microencapsulated antibiotic directly on the wound and suturing the skin closed. Groups of animals with treated wounds (ampicillin-loaded microcapsules), untreated wounds, wounds packed with unloaded microcapsules, and wounds packed with unencapsulated antibiotic were evaluated at daily intervals.

After the effectiveness of microcapsules A681-31-1 was established, a dose-response experiment was performed wherein doses of microcapsules ranging from 0.5 to 0.05 g were applied to wounds. Sixty-eight rats were divided into five groups (A through E); four groups of 15 rats and one group of 8 rats. All rats were infected on the same day with the same quantitated bacterial suspension to assure uniform inoculum in all wounds. Wounds in the group of 8 rats (Group A) were treated with 0.5 g of ampicillin microcapsules. Rats in Groups B, C, and D were treated with 0.25, 0.10, and 0.05 g, respectively. Rats in Group E remained untreated. Bacterial counts were performed on homogenized, preweighed tissue that was removed aseptically from the wound sites. Tissue from varying distances around the wound site and serum removed by cardiac puncture were assayed for antibiotic content. This was performed by placing disks saturated with known quantities of serum or tissue homogenates on the surface of Muller-Hinton agar previously seeded with standardized amounts of *Sarcina lutea* ATCC 9341. Following incubation at 37°C, inhibition zones were measured. Freshly diluted stock solutions containing known quantities of ampicillin anhydrate served as standards. Diameters of the inhibition zones were converted to antibiotic concentrations using standard curves generated by plotting the logarithm of the drug concentration against the

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zone diameters.

RESULTS AND DISCUSSION

The ampicillin microcapsules evaluated *in vivo* are listed in Table 1. The doses applied to each wound and the ampicillin core loadings (wt%) for each batch of microcapsules evaluated are shown in Table 2. With time, all microcapsules tested effectively reduced bacterial counts in contaminated wounds. However, microcapsules produced by the phase separation process were optimally effective in eliminating infection. An infection was considered eliminated when the wound site was bacteria free at 14 days. When 0.5 g of microcapsules (A382-140-1) were applied to wounds infected with *Staphylococcus aureus* and *Streptococcus pyogenes*, 60% of the wounds were sterile by 14 days. The remaining wounds were infected with *Staphylococcus aureus* only, since *Streptococcus pyogenes* was eliminated from all wounds by 48 hours. Wounds treated with an amount of powdered ampicillin equivalent to the core load amount, but not encapsulated within the DL-PLGA microcapsules, remained infected. Although 40% of the wounds remained contaminated at 14 days, the bacterial counts for these wounds were significantly lower than those observed for wounds treated with topical ampicillin powder or unloaded microcapsules (Table 3).

Results of the dose-response experiment performed to determine the smallest effective dose for microcapsules A681-31-1 are shown in Table 4. The bacterial counts listed in this table are for *Staphylococcus aureus* only, since all doses of microcapsules (A681-31-1) also eliminated *Streptococcus pyogenes* by 48 hours. At 7 days the wounds treated with encapsulated ampicillin remained infected with *Staphylococcus aureus*. By 14 days all wounds treated with encapsulated ampicillin were sterile; whereas, all untreated wounds remained infected. Doses of encapsulated ampicillin as small as 0.05 g per wound successfully eliminated *Staphlococcus aureus*. Based on the ampicillin core load, this quanity of microcapsule (0.05 g) contained approximately 9.05 mg (9050 μg) of ampicillin. If released uniformly over 14 days approximately 646 μg of ampicillin would be released into the wound. Kinetic studies of ampicillin released from C^{14} labeled ampicillin anhydrate microcapsules formulated by the phase separation process showed that only 60% of the total reservoir of ampicillin was released by 14 days. Considering this, approximately 387 μg of ampicillin was available for release per day. The amounts of ampicillin detected in muscle tissue removed from wounds treated with 0.05 g of microcapsule were 54, 60, and 21 μg/g of tissue at 2, 7, and 14 days respectively. This amount is theoretically more than adequate to effectively control the growth of *Staphylococcus aureus* since the minimal inhibitory concentration sufficient to kill 95% of all strains *in vitro* is 0.5 μg/ml. An *in vitro* ampicillin level as low as 0.05 μg/ml, or 10 times less, will inhibit 97% of all strains of *Streptococcus pyogenes*.

Microcapsules produced by the solvent evaporation process had low ampicillin core loadings (3.0-4.5 wt%). Kinetic studies showing the *in vitro* release of ampicillin from these microcapsules indicated that only 40% of the ampicillin was released by 14 days.(13) Nevertheless, these microcapsules eliminated infections and decreased bacterial counts when applied to infected wounds. However, even though large doses were applied (1.0-0.7 g microcapsule/wound) ampicillin was not detected in serum. Rats treated with high core loaded microcapsules produced by phase separation (A681-31-1) at a dose of 0.25 g/wound, had serum ampicillin levels present the first 4 days after treatment (Figure 2). Those treated with 0.5 g per wound had serum ampicillin levels 7 days post-treatment. No serum ampicillin was detected in rats treated with 0.10 g of microcapsule per wound or less.

The microcapsules currently evaluated have been formulated with 68:32 poly(DL-lactide-co-glycolide). The bioresorption time for unsterilized microparticles of that polymer is approximately 3-4 months *in vivo*. These microcapsules deliver drug at an efficacious rate over a target period of 2 weeks; however, they also release drug at a very slow rate for over 30 days following this initial 2 week period. Once infections in wounds are eliminated, the antibiotic and microcapsules are no longer wanted in the tissue. Therefore, new microcapsules consisting of 50:50 poly(DL-lactide-co-glycolide are being formulated. It is expected that these microcapsules will begin to biodegrade immediately following administration, and be completely degraded by 30 days. The more rapid biodegradation should reduce or eliminate the slow release of ampicillin that is occurring past 14 days.

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care, of the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council.

Commercial materials and equipment are identified in this report to specify the investigative procedures. Such identification does not imply recommendation or endorsement or that the materials and equipment are necessarily the best available for the purpose. Furthermore, the opinions expressed herein are those of the author and are not to be construed as those of the U. S. Army Medical Department.

ACKNOWLEDGMENTS

TABLE 1. *IN VIVO* AMPICILLIN MICROCAPSULES EVALUATED

CAPSULE NUMBER	MICROENCAPSULATION PROCESS	MICROCAPSULE SIZE, μ
9306-142-1	Solvent Evaporation	<250
A026-62-1	Solvent Evaporation	63-250
A382-140-1	Phase Separation	45-106
A681-31-1	Phase Separation	45-106

TABLE 2. AMPICILLIN CONTENT AND DOSE OF MICROCAPSULES APPLIED TO WOUNDS

IN VIVO EXPERIMENT	CAPSULE NUMBER	MICROCAPSULE DOSE/WOUND (AMPICILLIN EQUIVALENT)	ANTIBIOTIC CORE LOAD (WT%)
(EFFICACY)	9306-142-1	1.0 g (30.9 mg)	3
(EFFICACY)	A026-62-1	0.7 g (32.9 mg)	4.5
(EFFICACY)	A382-140-1	0.5 g (113 mg)	18.5
(DOSE-RESPONSE)	A681-31-1	0.50 g (110 mg) 0.25 g (45.25 mg) 0.10 g (18.10 mg) 0.05 g (9.05 mg)	18.1

TABLE 3. *STAPHYLOCOCCUS AUREUS* PRESENT IN WOUNDS FOLLOWING TREATMENT
WITH MICROCAPSULES A382-140-1, UNLOADED MICROCAPSULES, OR
UNENCAPSULATED AMPICILLIN

DAYS POST TREATMENT	MICROCAPSULES	UNLOADED MICROCAPSULES	UNENCAPSULATED AMPICILLIN
	(AVERAGE NUMBER* OF ORGANISMS PER GRAM OF TISSUE[+])		
2	$6.3 \pm 2.6 \times 10^6$	$1.3 \pm 1.5 \times 10^7$	$4.2 \pm 2.4 \times 10^6$
6	$4.7 \pm 2.2 \times 10^5$	$1.0 \pm 5.5 \times 10^6$	$5.3 \pm 7.0 \times 10^6$
8	$7.3 \pm 3.3 \times 10^4$	$4.5 \pm 9.7 \times 10^8$	$5.4 \pm 7.5 \times 10^6$
14	$9.4 \pm 0.6 \times 10^{3[§]}$	$4.7 \pm 9.8 \times 10^{5[×]}$	$1.8 \pm 3.2 \times 10^6$

* Mean $\pm$ Standard Deviation, n=5

[+] 6.0×10^9 *Staphylococcus aureus* inoculated/wound

[§] 3 of 5 wounds sterile

[×] Lowered due to competitive inhibition by superinfecting gram negative rods

When a regression curve was drawn using the data shown (log concentration of bacteria vs time) the linear component approached significance. This component represented the steady decrease in bacteria/gram of tissue observed in rats treated with microcapsules A382-140-1. When one outlier was removed from each group, significance ($p<0.05$) was observed.

TABLE 4. AVERAGE NUMBER OF *STAPHYLOCOCCUS AUREUS*/GRAM OF TISSUE PRESENT IN WOUNDS TREATED WITH DECREASING AMOUNTS OF MICROENCAPSULATED AMPICILLIN (A681-31-1)

GROUP	MICROCAPSULE DOSE (g)/WOUND	TOTAL AMPICILLIN AVAILABLE (mg)	Bacteria Per Gram of Tissue (mean ± S.D.,* n=5)		
			48 HOURS	7 DAYS	14 DAYS
A	0.5	90.50	$3.7 \pm 1.4 \times 10^6$	$7.7 \pm 10.0 \times 10^3$	0
B	0.25	45.25	$5.9 \pm 10.0 \times 10^3$	$8.2 \pm 14.0 \times 10^3$	0
C	0.10	18.10	$2.1 \pm 3.0 \times 10^6$	$1.8 \pm 5.4 \times 10^4$	0
D	0.05	9.05	$6.8 \pm 4.7 \times 10^5$	$4.4 \pm 3.8 \times 10^4$	0
E	0.00	0.00	$1.8 \pm 1.8 \times 10^6$	$3.5 \pm 4.3 \times 10^6$	$6.4 \pm 9.6 \times 10^5$

*Standard Deviation
1.5×10^{10} *S. aureus* inoculated/wound
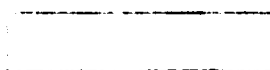
Figure 1.   Ampicillin Concentrations Detected in Homogenized Muscle Tissue (Deep)
at 2, 7, and 14 Days Following Wound Site Treatment   with
Microencapsulated Ampicillin (A 681-31-1)
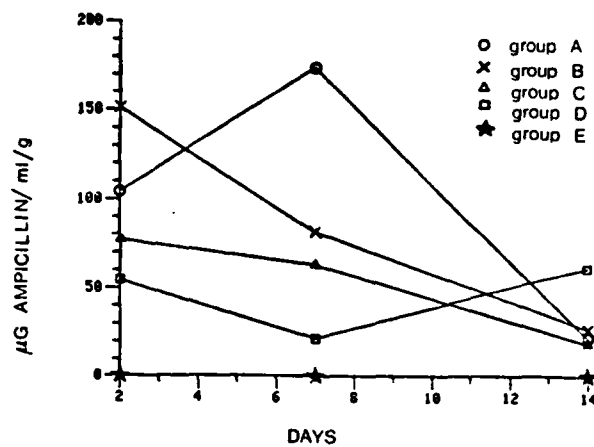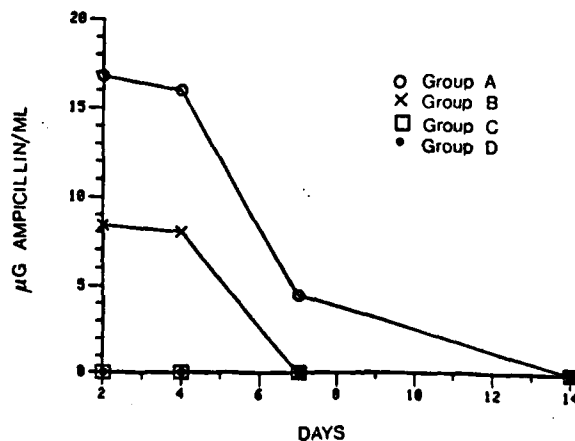

Figure 2.   Ampicillin Concentrations Detected in Serum at 2, 4, 7, and 14 Days
Following the Application of Microencapsulated Ampicillin
(Batch A 681-31-1) to Infected Wounds

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